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We have developed flow cytometric methods for the analysis of androgen and vitamin D receptor expression in nuclei isolated from archival breast tumors of both male and female patients. Our data shows that in male breast tumors androgen receptor expression is less than that of the female tumors. In both male and female tumors, sub-populations with diploid DNA content had lower receptor expression than in nuclei with aneuploid DNA content. In contrast the expression of vitamin D receptors in diploid and aneuploid tumors was similar. The methods developed are rapid, quantitative and allow for multiparametric analysis of receptor expression in archival breast tumors.

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INTRODUCTION: Flow cytometry (FCM) is an important method for monitoring of cellular receptor expression in hematological tumors and for the determination of DNA content and proliferation in breast tumors. However, use of FCM for monitoring hormone receptor expression in breast tumors has been limited, mostly due to the difficulty in obtaining single cell suspensions and reduced immunoreactivity of the receptor antigens in formalin fixed tissues. Our project is focused on developing flow cytometric methods for multiparametric determination of hormone receptor expression in human breast tumors. Our earlier work has focused on estrogen and progesterone receptors (1,2), and more recently we have concentrated our efforts on the study of androgen and vitamin-D receptors (3,6,7) in archival tumors. Our flow cytometric methods are rapid, highly sensitive and can determine not only the percentage of receptor positive cells in a heterogeneous population but also measure antigen density on the individual cells and subpopulations in relation to their DNA content. We believe flow cytometric analysis of hormone receptors and other cellular markers can be an important quantitative and multiparametric method for diagnostic and prognostic studies in breast cancer.

BODY:

1. Androgen, Vitamin–D Receptor Expression and DNA Content of Human Breast Tumors: Specific aims of the year 02 were to refine methods for enzymatic digestion, antigen retrieval and immunostaining of archival breast tumor biopsies for the analysis of androgen and Vitamin-D receptors and DNA content.

During 02 yr, archival formalin fixed-paraffin embedded breast tumors were collected from the Pathology Department Repository of the Jackson Memorial Medical Center, Miami. Our co-investigator, Dr. M. Nadji had sections cut for histopathological analysis and selected material for flow cytometric analysis. Thick (50 micron) and thin (5 micron) sections were cut from seventy breast tumor samples for analysis by immunohistochemistry and laser flow cytometry. Thick sections were enzymatically digested, processed for antigen retrieval and studied for androgen and Vitamin-D receptor expression and DNA content (aneuploidy and cell cycle distribution) by flow cytometry. Thin sections cut were stained by immunohistochemistry and studied for ER expression and histopathological grading.

Data from immunohistochemistry and flow cytometry was analyzed for percentage of androgen and Vitamin-D positive cells and DNA content of the subpopulations. List mode data was collected for gated analysis of receptor expression and DNA ploidy. Correlation between receptor expression and DNA ploidy of the sub-populations was determined. Data was presented at two national meeting (American Association for Cancer Research and International Society for Analytical Cytology). An abstract has been submitted for presentation at the Department of Army meeting in Orlando, Fl. A manuscript based on these findings is in final stages of being readied for publication (8).

2. Multiparametric Analysis of Nuclear Volume and DNA Content of Nuclei Isolated from Fresh Human Breast Tumor Biopsies:

We have earlier described the development and use of a high-resolution flow cytometer, which can simultaneously measure nuclear volume and DNA content of nuclei in suspension

(4). Our published data showed that this instrument by using multiparametric analysis of electronic nuclear volume versus DNA content can differentiate between normal and tumor cells and identify tumor cells in secondary sites such as lymph nodes or bone marrow of breast tumor patients (5). In the 02-year we processed 38 fresh breast tumor biopsies for multiparametric analysis of nuclear volume vs. DNA content in this instrument. The major goal of these investigations was to see if the ratio of nuclear volume to DNA content could be used for differentiating between normal and tumor cells. Although we generated high resolution histograms of nuclear volume vs. DNA content of a variety of breast tumors with diploid and aneuploid sub-populations, our data did not support the hypothesis that ratio of nuclear volume to DNA content can be used universally to differentiate between normal and tumor cells in a heterogeneous population of normal and tumor cells from a breast tumor biopsy. Several technical problems in the measurement of nuclear volume by Coulter principal became evident during these investigations. On the basis of feed back from these studies, several modifications were made in the NASA/ACS flow cytometer. The upgraded and modified instrument is capable of performing reliable nuclear volume vs. DNA content measurements. We intend to reexamine the breast tumor specimens analyzed earlier to test the hypothesis earlier proposed.

KEY RESEARCH ACCOMPLISHMENTS:

- Collection and analysis of breast tumor blocks from 25 female and 34 male patients were processed for the expression of androgen and vitamin D receptor expression.

- The percentage of Androgen receptor positive cells in diploid female breast tumors varied from a low of 28 to as high as 89 with a mean and standard deviation of 55 ± 19. In triploid sub-populations, the range was from 33 to 96 with mean of 63 ± 17. In tumor populations with greater than 4N DNA content, the range of Androgen positive cells was from 51 to 98 with a mean and standard deviation of 85 ± 18.

- Antigen density measurements indicated that the MFC (mean fluorescence channel ratio of isotype vs. antibody stained nuclei) of diploid nuclei in female breast tumors varied from 1.87 to 6.5. In tumors with triploid subpopulations, the MFC ratio was 2.28 to 6.80 while in the near tetraploid tumors the ratio was from 2.3 to 6.79.

- Of the thirty-four human male breast tumors analyzed, the percent of androgen receptor positive diploid nuclei varied from 5 to 61 with mean of 31 ± 14. In triploid sub-populations, the range was from 23 to 64 with a mean of 44 ± 15. In sub-populations with greater than 4N DNA content, the range was from 16 to 61 with a mean value of 40 ± 16.

- In nuclei from diploid tumors, the percent of vitamin D receptor positive cells

varied from 28 to 86 with mean of 66 ± 21. In triploid tumors the range was 68 to 91 with a mean of 70 ± 16. In sub-populations with greater than 4N DNA content the range was from 36 to 91 with mean of 63 ± 20.

REPORTABLE OUTCOMES:

Gated analysis of nuclei isolated from paraffin blocks of tumors obtained from female and male patients showed that the percentage of androgen receptor positive cells was in general lower in male breast tumors than in the female tumors. In both the male and the female tumors, the percentage of androgen receptor positive cells increased with increases in DNA ploidy; the hyper-diploid (triploid, tetraploid) populations having more androgen positive cells than the diploid populations.

In contrast to androgen receptor expression, Vitamin-D receptor expression did not increase with increase in ploidy of nuclei isolated from female breast tumors

The flow cytometric methods developed in our laboratory can be used for rapid quantitation of androgen and vitamin- D receptor expression in formalin fixed/paraffin embedded breast tumors.

PRESENTATION AT NATIONAL MEETINGS:

Abstracts and posters on different aspects of our work on "Flow Cytometric Analysis of Androgen and Vitamin D Receptor Expression in Breast Tumors" were presented by invitation at 1. The Advanced Flow Cytometry Symposium held by the International Society for Optical Engineering, (San Jose January 2002). Two other posters and abstracts on hormone receptor expression in human breast tumors were presented at the Annual Meeting of the American Association for Cancer Research (San Francisco, April 2002), and at the Annual meeting of the International Society for Analytical Cytology (San Diego, April, 2002).

Techniques developed in our laboratory were also demonstrated at the First Indo-US Workshop on Advanced Flow Cytometry (Feb, 2002) held in Chandigarh, India. Forty-six students interested in use of flow cytometry for analysis of tumor cells attended this 6-day workshop. A lecture on procedures developed under the auspices of this grant was followed by hands-on demonstration of measuring estrogen and androgen receptor expression in archival human breast tumors.

International Union Against Cancer (UICC) has awarded an ICRETT (technology transfer) fellowship for three month's visit of Dr. Rao, Associate Professor in Radiation Oncology, KS Medical School, Manipal, India to study hormone receptor expression in archival breast tumors collected from the Indian patients.

CONCLUSIONS: Our data shows that flow cytometric analysis of androgen and vitamin D

expression in archival formalin fixed paraffin embedded breast tumor samples can be a useful technique for retrospective studies seeking to correlate hormone receptor expression with clinical outcome. The major advantages of the methods we have developed are that they are quantitative and can simultaneously determine expression of different hormonal receptors in the nuclei as well as determine their DNA content. This multiparametric capability will allow for analysis of individual sub-populations in a heterogeneous tumor cell population consisting of diploid normal and aneuploid tumor cells. The combination of nuclear volume measurement with other cellular markers such as DNA and/or hormone receptors by flow cytometry is a powerful technique for analysis of tumor cells. We are preparing submission of three manuscripts based on work performed with support from this grant.

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APPENDICES:

Figure 1. Androgen Receptor Expression in Female Breast Tumors.
Figure 2. Androgen Receptor Expression in Male Breast Tumors.
Figure 3. Vitamin D Receptor Expression in Female Breast Tumors.

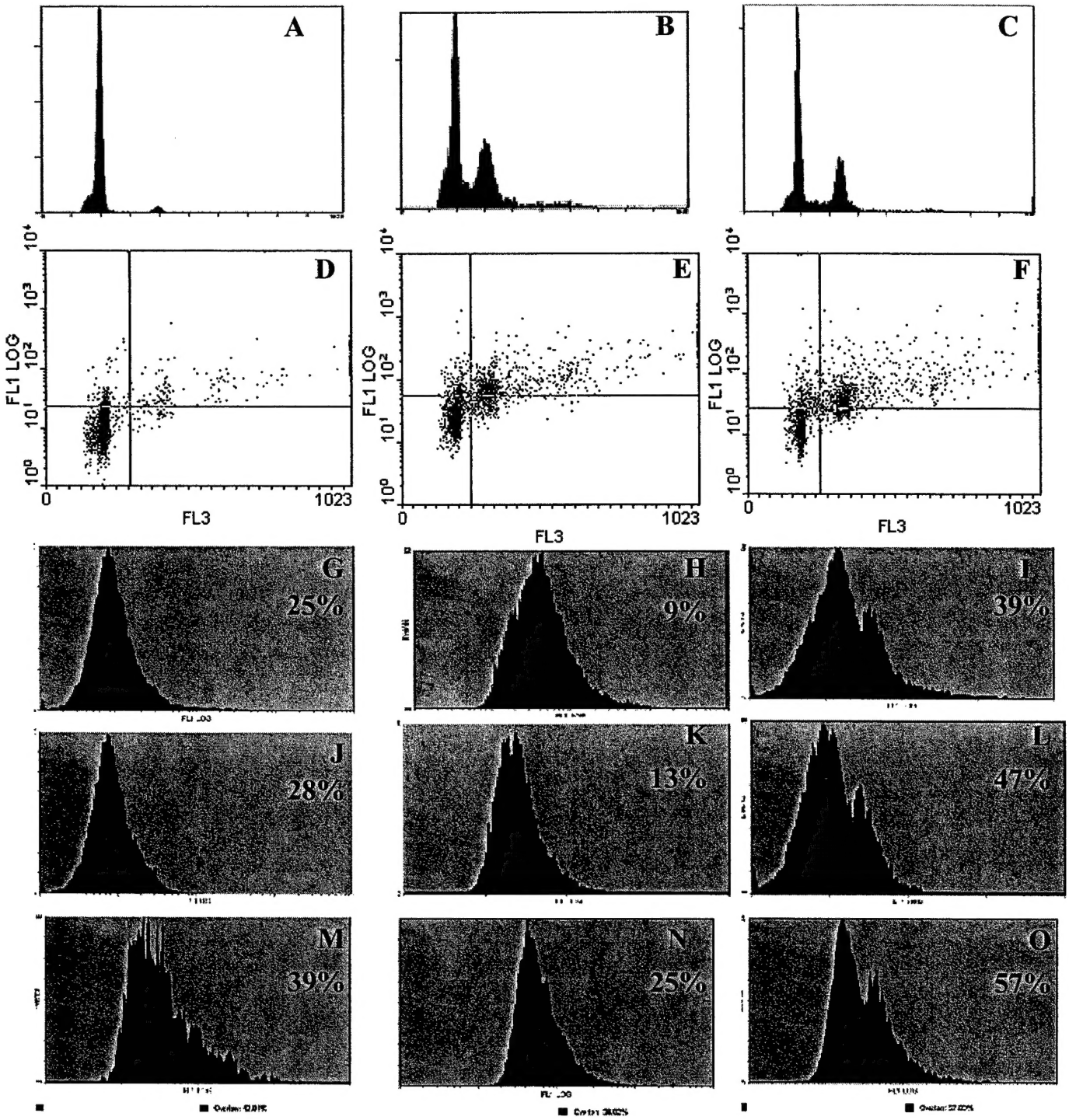

Figure 1. Androgen Receptor Expression in Female Breast Tumors. Histograms (A-C) are representative of tumors with diploid (A), triploid (B) and hypo-tetraploid (C) subpopulations. The scatter plots (D-F) show DNA content (X-axis) vs. androgen receptor expression (Y axis) of nuclei. The overlay histograms in the lower three rows show analysis of the isotype and the androgen antibody treated samples. Overlays in the first row are of the total nuclear population while those in the middle and bottom are of gated sub-populations with diploid and aneuploid DNA content, respectively. In general these selected overlays show that the aneuploid sub-populations had higher androgen receptor expression than the sub-population with diploid DNA content.

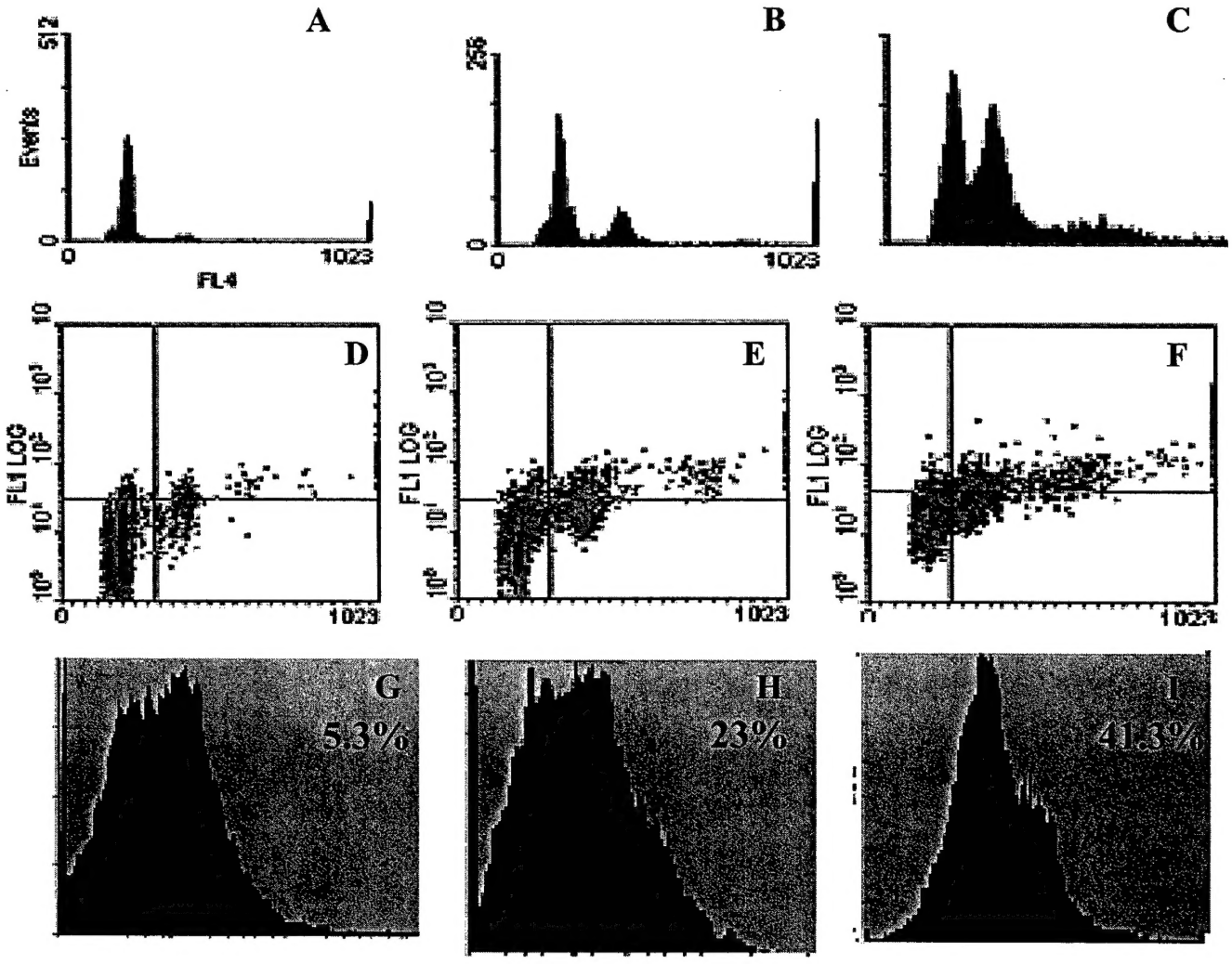

Figure 2. Androgen Receptor Expression in Male Breast Tumors. Histograms (A-C) show representative DNA histograms of breast tumors from male patients with diploid and aneuploid subpopulations. The scatter plots (D-F) show DNA content (X-axis) vs. androgen receptor expression (Y axis) of nuclei incubated with the anti-androgen antibody. Dots above the horizontal line indicate nuclei with positive receptor expression. The overlay histograms in Fig 3 (G-I) show analysis of the isotype vs. antibody treated samples by the Overton's method.

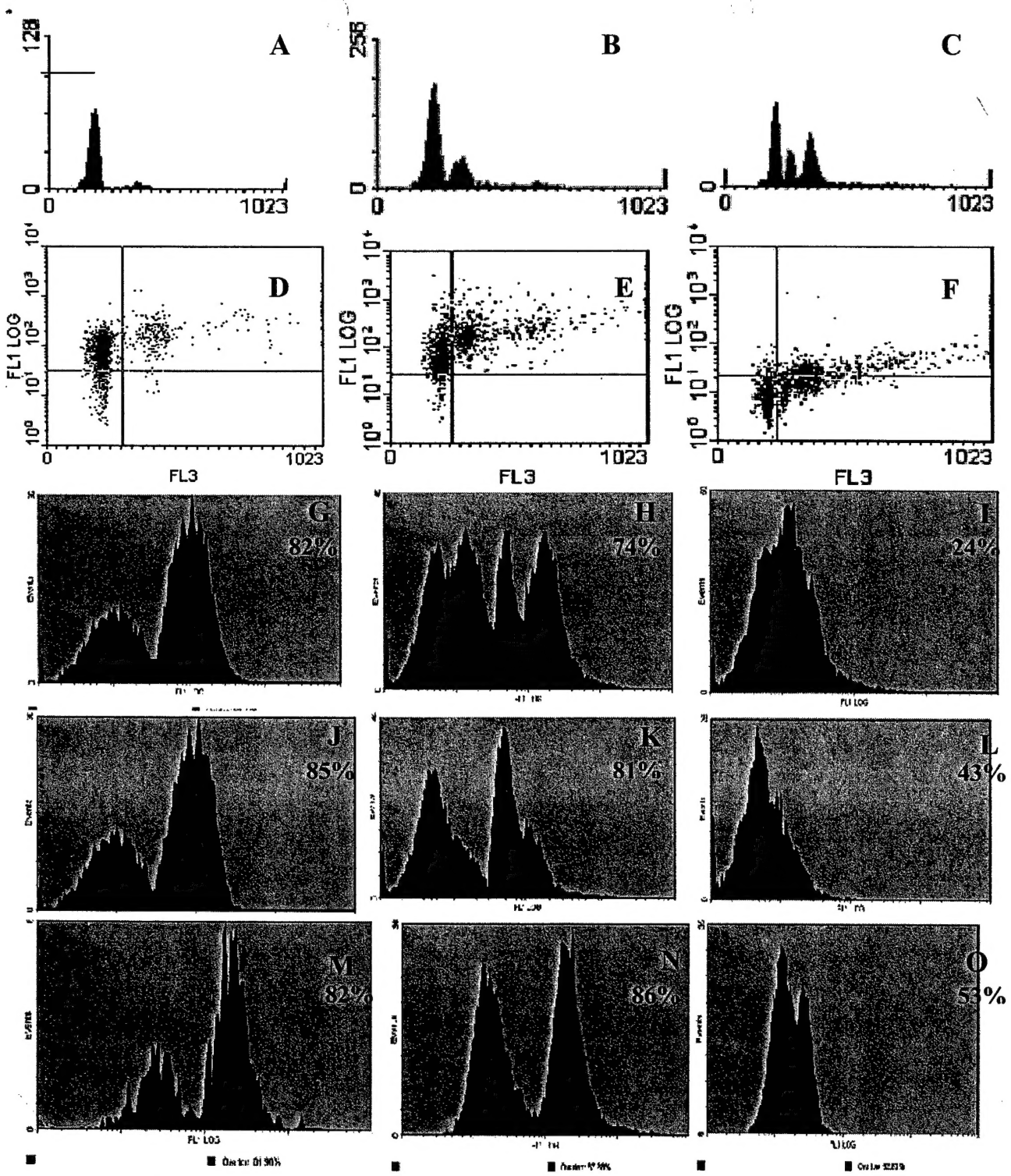

Figure 3. Vitamin D receptor expression in Breast Tumors: DNA histograms (A-C) show breast tumor with diploid, triploid and multiploid sub-populations. The overlay histograms (G-O) are from the analysis of the isotype vs. Vitamin D antibody treated samples of the total population (first row), the diploid population (middle row) and of the aneuploid population (bottom row). In general Vitamin D receptor expression of the diploid and aneuploid tumor sub-populations was similar.